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CRYOPRESERVED PRIMARY HEPATOCYTES AS A CONSTANTLY AVAILABLE IN VITRO MODEL FOR THE EVALUATION OF HUMAN AND ANIMAL DRUG METABOLISM AND ENZYME INDUCTION*

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ABSTRACT

The use of primary hepatocytes is now well established for both studies of drug metabolism and enzyme induction. Cryopreservation of primary hepatocytes decreases the need for fresh liver tissue. This is especially important for research with human hepatocytes because availability of human liver tissue is limited. In this review, we summarize our research on optimization and validation of cryopreservation techniques.

The critical elements for successful cryopreservation of hepatocytes are (1) the freezing protocol, (2) the concentration of the cryoprotectant [10% dimethylsulfoxide (DMSO)], (3) slow addition and removal of DMSO, (4) carbogen equilibration during isolation of hepatocytes and before cryopreservation, and (5) removal of unvital hepatocytes by Percoll centrifugation after thawing. Hepatocytes of human, monkey, dog, rat, and mouse isolated and cryopreserved by our standard procedure have a viability $\geq$ 80%. Metabolic capacity of cryopreserved hepatocytes determined by testosterone hydroxylation, 7-ethoxyresorufin-*O*-deethylase (EROD), 7-ethoxycoumarin-*O*-deethylase (ECOD), glutathione *S*-transferase, UDP-glucuronosyl transferase, sulfotransferase, and epoxide hydrolase activities is $\geq$60% of freshly isolated cells. Cryopreserved hepatocytes in suspension were successfully applied in short-term metabolism studies and as a metabolizing system in mutagenicity investigations. For instance, the complex pattern of benzo[*a*]pyrene metabolites including phase II metabolites formed by freshly isolated and cryopreserved hepatocytes was almost identical. For the study of enzyme induction, a longer time period and therefore cryopreserved hepatocyte cultures are required. We present a technique with cryopreserved hepatocytes that

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allows the induction of testosterone metabolism with similar induction factors as for fresh cultures. However, enzyme activities of induced hepatocytes and solvent controls were smaller in the cryopreserved cells.

In conclusion, cryopreserved hepatocytes held in suspension can be recommended for short-term metabolism or toxicity studies. Systems with cryopreserved hepatocyte cultures that could be applied for studies of enzyme induction are already in a state allowing practical application, but may be further optimized.

I. INTRODUCTION

For several applications, intact hepatocytes have been shown to be superior over, for instance, microsomal fractions of liver homogenate. One example is that the use of intact hepatocytes in mutagenicity tests improved the correlation between mutagenicity and carcinogenicity data. This has been shown in a study that examined the correlation between rodent carcinogenicity and mutagenic efficiency of several polycyclic aromatic hydrocarbons in the Ames test using either intact rat hepatocytes or equivalent amounts of NADPH-fortified homogenized rat hepatocytes as metabolizing systems (Figs. 1 and 2) [1]). In this study, benzo[*a*]pyrene and the 12 monomethylbenzo[*a*]pyrenes were examined in the Ames test. These benzo[*a*]pyrene derivatives differ in rodent carcinogenicity from very potent to apparently inactive (Fig. 1). Using hepatocyte homogenate, there was no correlation between the mutagenic efficacy and the tumor-initiating activity in rats. When hepatocyte homogenate was replaced by an equivalent amount of intact hepatocytes, the mutagenicity of all benzo[*a*]pyrene derivatives was reduced; however, the extent of reduction was small in the case of the potent tumor initiators, but it was marked with

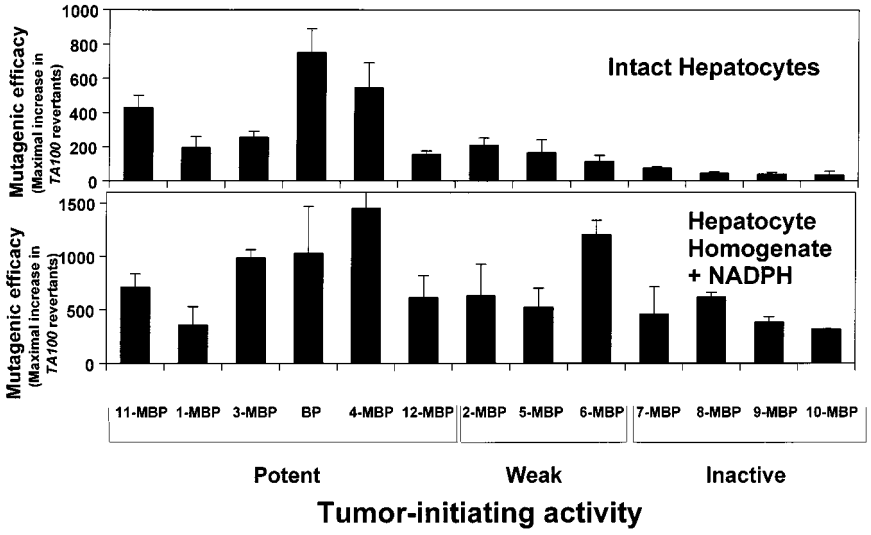

FIG. 1. Mutagenicity of benzo[*a*]pyrene (BP) and the 12 monomethylbenzo[*a*]-pyrenes (MBP) using intact hepatocytes and NADPH-fortified hepatocyte homogenate as metabolizing systems. (From Ref. 1.)

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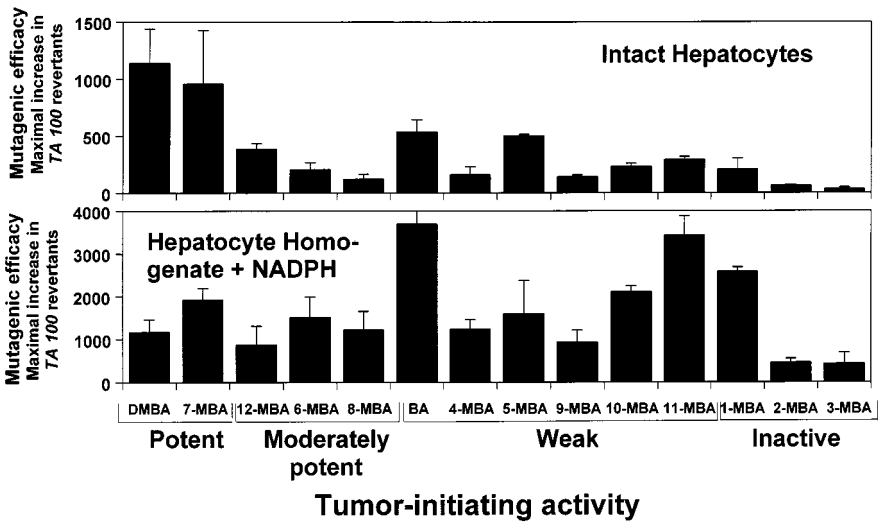

FIG. 2. Mutagenicity of benzo[*a*]anthracene (BA), the 12 monomethylbenz[*a*]anthracenes (MBA), and 7,12-dimethylbenz[*a*]anthracene (DMBA) using intact hepatocytes and hepatocyte homogenate as metabolizing systems. (From Ref. 1.)

the noncarcinogens. Thus, the use of intact hepatocytes diminished discorrelations to the available carcinogenicity data. A similar improvement by intact hepatocytes was also observed for other substances {e.g., for benzo[*a*]anthracene and its monomethyl derivatives (Fig. 2 [1])}. Two mechanisms are major contributors to the differences between homogenate-mediated and cell-mediated mutagenicity: (1) a deficiency of phase II metabolism in homogenate due to dilution of cofactors and (2) loss of barrier effects due to disruption of the plasma membrane for cell homogenate.

The use of primary hepatocytes is now well established for both pharmacological and toxicological studies. Numerous favorable reports on the correlation of such in vitro data with primary hepatocytes to the in vivo situation have been reported [2–4; reviews in Refs. 5 and 6]. One of the main problems in toxicology is interspecies extrapolation, particularly from laboratory animals to man. Thus, human hepatocytes are of especially high interest. Because human hepatocytes are only occasionally available—but then sometimes in large amounts up to 5 billion hepatocytes [5]—we optimized cryopreservation techniques to minimize alterations of xenobiotic metabolizing enzymes. Several laboratories have reported successes in cryopreservation of freshly isolated hepatocytes in the past decade [7–16]. In the present review, we summarize our research on optimization and validation of cryopreservation techniques and present some applications of cryopreserved hepatocytes.

II. OPTIMIZATION OF PARAMETERS THAT INFLUENCE THE QUALITY OF CRYOPRESERVATION

One of the main parameters that influence the quality of cryopreservation is the freezing protocol. Hepatocytes from male Sprague–Dawley rats isolated by a modified

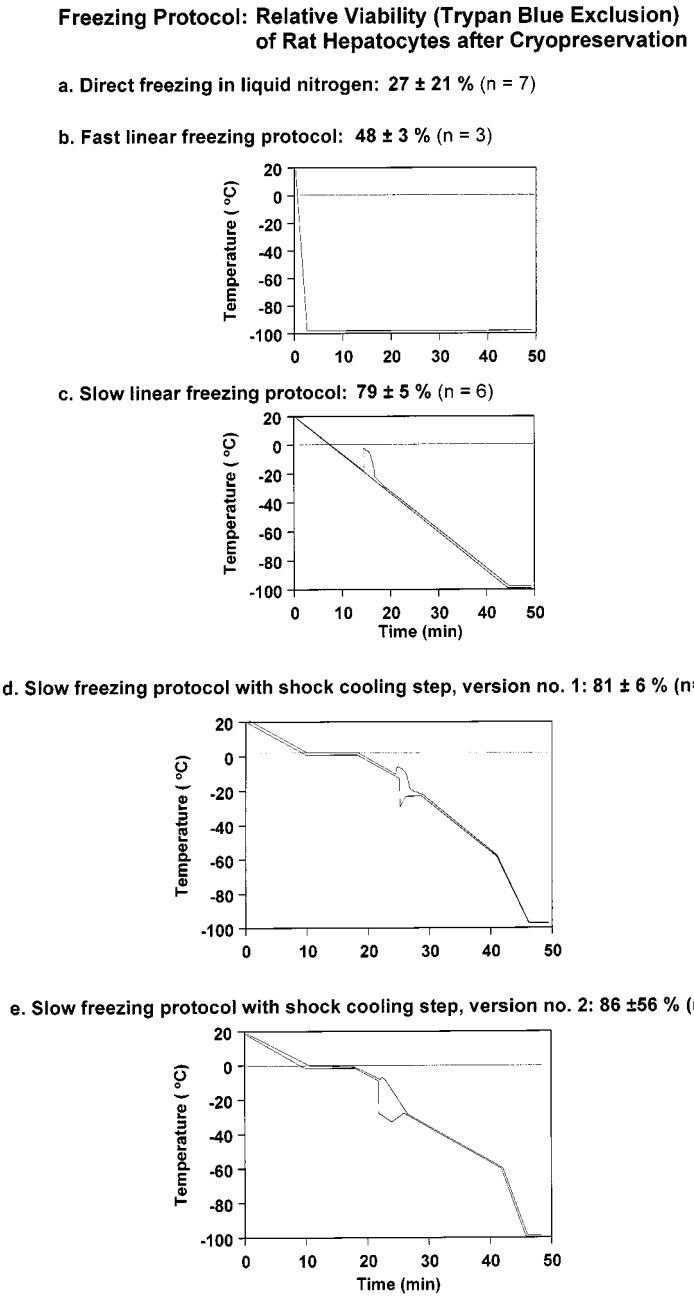

FIG. 3. Influence of the freezing protocol on viability (Trypan Blue exclusion) of rat hepatocytes after cryopreservation. Five different freezing protocols were tested: (a) direct freezing in liquid nitrogen; (b) fast linear freezing protocol (−38°C/min); (c) slow linear freezing protocol (−2°C/min); (d) slow freezing protocol with shock cooling step, version 1; (e) slow freezing protocol with shock cooling step, version 2. All freezing protocols included 10% DMSO as a cryoprotectant, a cell concentration of 4×10^6 hepatocytes per cryovial, and gradual addition and removal of DMSO. (From Ref. 10.)

two-step collagenase perfusion technique were shock frozen in liquid nitrogen. Despite the presence of 10% of DMSO as a cryoprotectant, viability determined after thawing was only 27% (Fig. 3a [10]). A fast freezing protocol with a cooling rate of −38°C/min resulted in a viability of 48% (Fig. 3b). However, using a slow freezing protocol with only −2°C/min led to much better viabilities (Fig. 3c). Using the latter freezing protocol, we determined the temperature in the freezing chamber (lower line) as well as in the cell suspension of the cryovial (upper line), showing that the cell suspension becomes supercooled (Fig. 3c). When crystallization starts, the latent heat of fusion is suddenly released and the cell sample is warmed. Because this release of heat may be deleterious, we developed programs to minimize warming by the introduction of a shock cooling step. For the cooling program shown in Fig. 3d, shock cooling was initiated a little too late, whereas the program shown in Fig. 3e almost completely compensates for crystallization heat, resulting in an additional improvement of viability.

Another important parameter is the concentration of cryoprotectant. In our hands, 10% of DMSO have been shown to be optimal (Fig. 4 [10]). Higher concentrations of DMSO are toxic due to high osmolarities.

Two minor parameters of cryopreservation quality are the number of cells per cryovial—we usually freeze 4×10^6 hepatocytes per cryovial—and the technique of addition and removal of DMSO (Fig. 5). It should be considered that a cell suspension with 10% DMSO has an osmolarity about five times higher than under physiological conditions. Thus, gradual addition and gradual removal of the cryoprotectant allows the cells to adapt more efficiently to osmotic changes, causing a small but significant increase in survival rate.

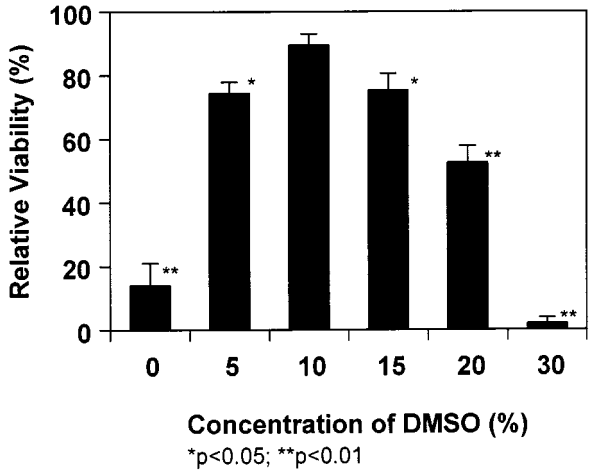

FIG. 4. Relative viability (Trypan Blue exclusion) of rat hepatocytes after cryopreservation, using the optimized freezing protocol with 4×10^6 cells/vial and gradual addition and removal of DMSO. The difference in viability between samples with 10% DMSO and all other DMSO concentrations shown was statistically significant. The data are mean values and standard deviations from three independent experiments. (From Ref. 10.)

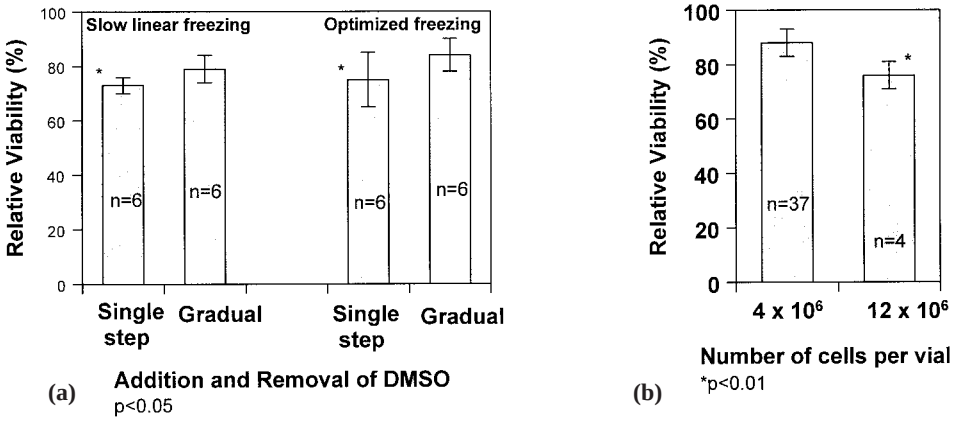

FIG. 5. Influence of cell number per vial (a) and gradual versus single-step removal of DMSO (b) on relative viability (Trypan Blue exclusion) of rat hepatocytes. (a) Gradual addition of DMSO: addition of an equal volume of 4% DMSO followed by the addition of an equal volume of 16% DMSO after 5 min on ice. Gradual removal of DMSO: addition of 0.5, 1, 2, 3, and 6 mL of ice-cold buffer to 1 mL of cell suspension at 3-min intervals. The influence of gradual versus single-step addition and removal of DMSO was examined for rat hepatocytes cryopreserved by a slow linear freezing protocol (−2°C/min) and by the optimized freezing protocol (slow freezing protocol with shock cooling step, version 2). (From Ref. 10.)

III. VIABILITY AND PHASE I METABOLISM OF CRYOPRESERVED HEPATOCYTES HELD IN SUSPENSION

For several studies, suspensions of hepatocytes are used, for instance, to analyze the metabolism of new drugs or as metabolizing systems in mutagenicity tests. Therefore, we compared viability and metabolic capacity of cryopreserved and freshly isolated hepatocytes held in suspension for up to 2 h. The species examined in our laboratory are human, monkey (male cynomolgus), dog (male Beagle), rat (male Sprague–Dawley), and mouse (male NMRI). The hepatocytes were cryopreserved and thawed as described [13,16] and DMSO was gradually removed. Incubations were performed in a buffer containing amino acids and glucose, but without hormonal additives in a shaking water bath at 37°C and analyses were performed after 30, 60, and 120 min [13]. After the respective incubation periods, hepatocytes were homogenized and all enzyme assays were performed with NADPH-fortified hepatocyte homogenate. Thus, the applied assays indicate whether the respective enzymes of the cryopreserved hepatocytes are still active, but they do not give information on *in situ* availability of cofactors, such as NADPH.

Human hepatocytes were obtained from liver tissue of seven patients who underwent surgery because of liver metastasis, mostly due to colon cancer. Viability determined by lactate dehydrogenase (LDH) release was only slightly decreased in cryopreserved compared to freshly isolated hepatocytes and only a very small decrease was observed during the 2-h incubation period (Fig. 6). Similarly, albumin secretion determined by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies specific for human albumin was quite constant in the cryopreserved cells.

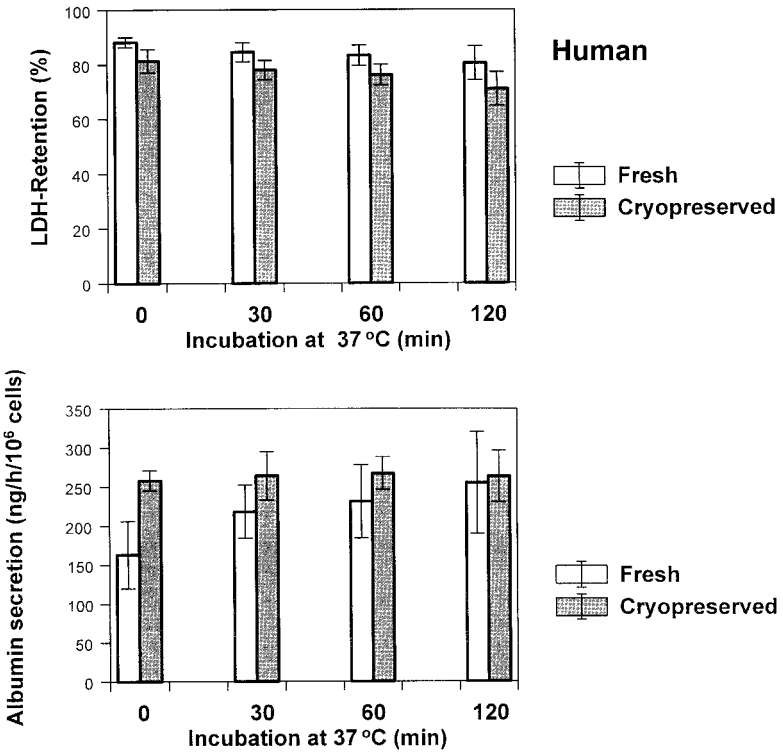

FIG. 6. Viability determined by LDH retention and albumin secretion of cryopreserved versus freshly isolated human hepatocytes. The data represent mean values and standard deviations of hepatocytes obtained from seven patients. (From Ref. 13.)

O-Deethylation of 7-ethoxyresorufin (EROD) is catalyzed by CYP1A1 and 1A2 [17]. There was no reduction in EROD activities in cryopreserved cells during the incubation period of 2 h (Fig. 7). EROD activities were smaller in cryopreserved compared to freshly isolated hepatocytes and fresh liver homogenate. However, the difference should still be acceptable for metabolic studies, whereby we define activities as ''acceptable for metabolic studies'' if the values of cryopreserved cells are higher or equal to 60% of the freshly isolated hepatocytes.

O-Deethylation of 7-ethoxycoumarin (ECOD) can be catalyzed by a wide range of cytochrome P-450 forms, including CYP1A1, 1A2, 2A1, 2B1, 2B2, 2B6, 2C6, 2C7, 2C11, 2C13, and 2E1 [18]. A decrease of about 40% was observed for cryopreserved hepatocytes, but this was also the case for the freshly isolated cells (Fig. 7).

Total hydroxylation of testosterone, determined by high-performance liquid chromatography (HPLC) [19], was relatively constant in cryopreserved human hepatocytes and in a similar range as for freshly isolated hepatocytes and even for human liver homogenate (Fig. 8). A deficiency of some previous studies with cryopreserved hepatocytes is that data on phase I enzymes of freshly isolated and cryopreserved hepatocytes but no data on the respective fresh liver homogenate have been shown. Thus, it cannot be excluded that although data of cryopreserved and fresh hepatocytes are similar, enzyme activity has been lost during the cell-isolation procedure. Similarly, several studies with hepatocyte

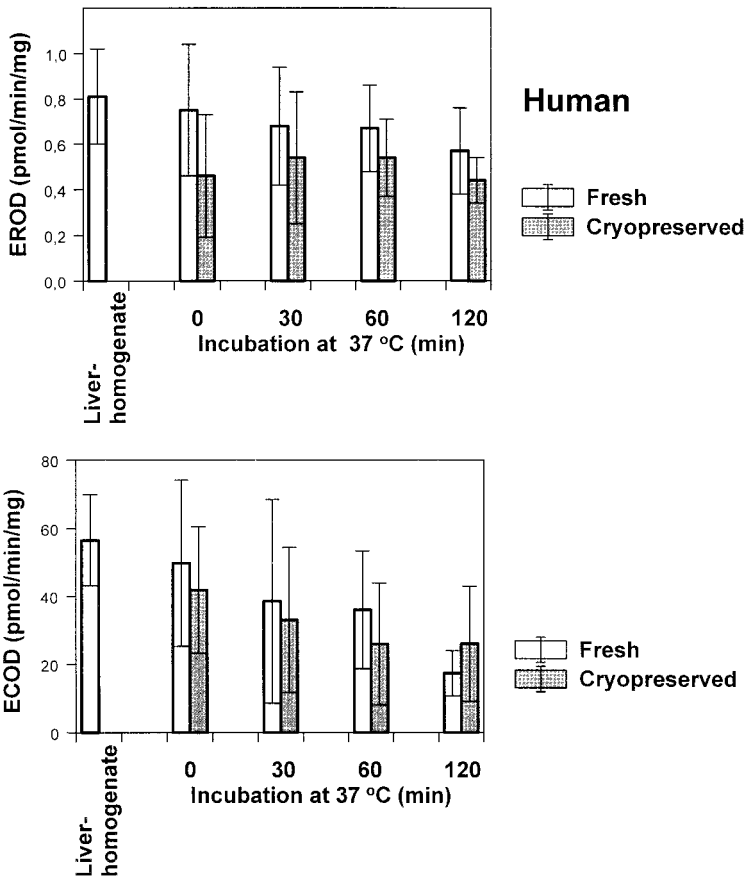

FIG. 7. EROD and ECOD activities in freshly isolated and cryopreserved human hepatocytes. The data represent mean values and standard deviations of hepatocytes obtained from seven patients. (From Ref. 13.)

cultures have shown a stable expression of various phase I enzymes for several days or even weeks, but it may be suspected that this stable expression occurs on a much lower level than in vivo. To exclude such a bias, we present data of fresh liver homogenate for all examinations of phase I enzymes [13]. The main testosterone metabolites formed by human hepatocytes are the 6β- and 2β-testosterone hydroxylation products (OHT) (Table 1). 6β-OHT is formed mainly by CYP3A4 and so is 2β-OHT, but 2β-OHT is also formed by CYP1A1. In cryopreserved cells, both activities are in a range that is acceptable for metabolic studies.

Similar experiments were performed with suspended hepatocytes of rats and mice. Cryopreserved hepatocytes of male Sprague–Dawley rats had an acceptable viability (Fig. 9) and EROD and ECOD activities (Fig. 10), although there is a decrease in ECOD activity for both freshly isolated and cryopreserved cells during the incubation time (Fig. 10).

Testosterone metabolism by rat hepatocytes is much more complex compared to humans, with 16α-, 2α-, and 6β-OHT representing the main metabolites (Table 2). CYP2B1 and 2C11 are responsible for formation of 16α-OHT, 2α-OHT is predominantly

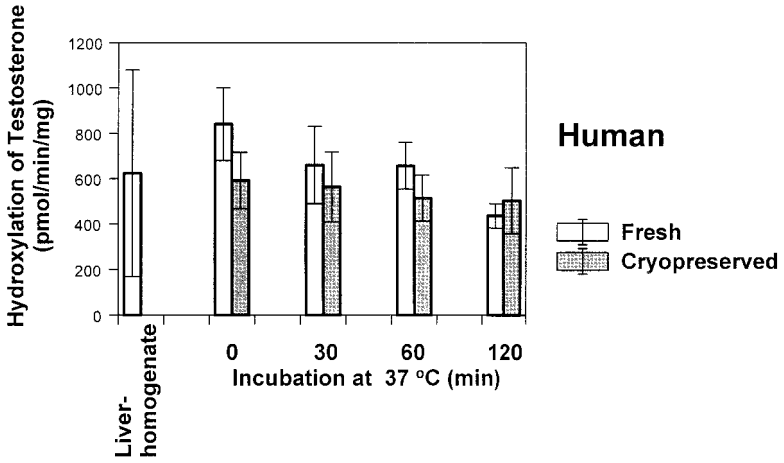

FIG. 8. Total hydroxylation of testosterone in freshly isolated and cryopreserved human hepatocytes. The data represent mean values and standard deviations of hepatocytes obtained from seven patients. (From Ref. 13.)

TABLE 1

Formation of Hydroxylated Testosterone Metabolities (pmol/min/mg Protein) in Freshly Isolated and Cryopreserved Human Hepatocytes in Suspension

Metabolite	0 min	30 min	60 min	120 min
		Freshly Isolated Cells		
6β-OHT	626 ± 157	517 ± 155	525 ± 57	335 ± 52
2β-OHT	76 ± 18	62 ± 15	67 ± 11	39 ± 5
		Cryopreserved Cells		
6β-OHT	468 ± 124	442 ± 136	398 ± 71	405 ± 179
2β-OHT	62 ± 19	54 ± 14	51 ± 12	47 ± 24

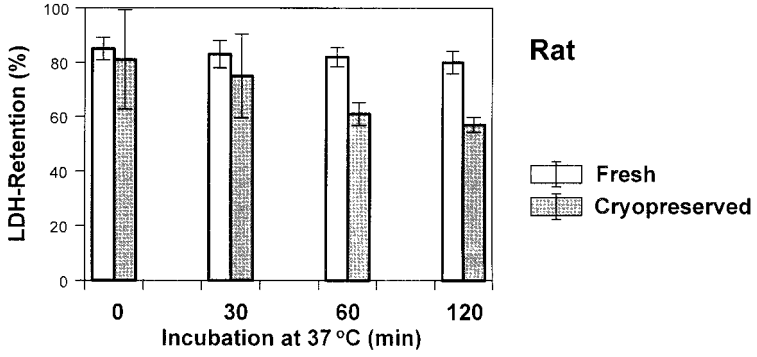

FIG. 9. Viability determined by LDH retention of cryopreserved versus freshly isolated rat hepatocytes. The data represent mean values and standard deviations of hepatocytes obtained from four rats. (From Ref. 13.)

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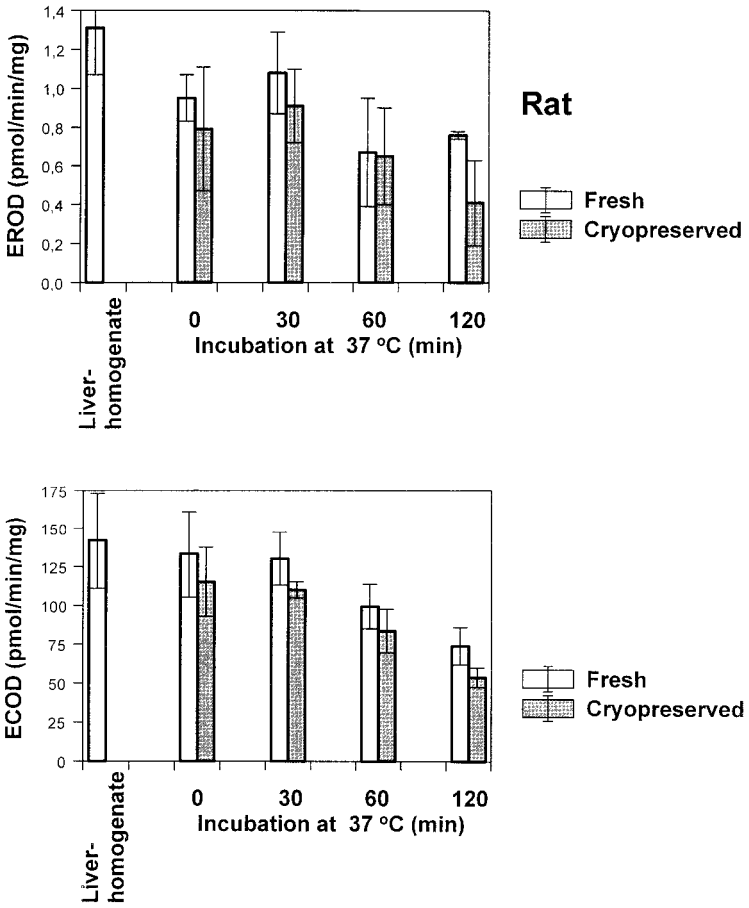

FIG. 10. EROD and ECOD activities in freshly isolated and cryopreserved rat hepatocytes. The data represent mean values and standard deviations of hepatocytes obtained from four rats. (From Ref. 13.)

formed by CYP2C11 (the isoenzyme specific for the male rat), and 6β-OHT predominantly by 3A1. The data show no major loss of activity for the cryopreserved cells (Fig. 11) not even for metabolites that are formed only to a minor extent (Table 2) [13].

For analysis of mouse hepatocytes (male NMRI mice were used), we had to pool hepatocytes from six individuals, because the number of hepatocytes obtained from one mouse is relatively low. However, concerning the quality of cryopreserved cells, the mouse seems to be an unproblematic species [13]. Using mouse hepatocytes, we observed no significant decrease in LDH release (Fig. 12), a good maintenance of EROD activity (Fig. 13), a decrease in ECOD activity for both freshly isolated and cryopreserved hepatocytes (Fig. 13), similar to that seen for rat and human hepatocytes, and also acceptable data for testosterone metabolism (Fig. 14; Table 3). Mouse hepatocytes produced a different pattern of testosterone hydroxylation products when compared to human and rat, with 6β-OHT representing the main metabolite. Nevertheless, very similar results were obtained for freshly isolated and cryopreserved hepatocytes.

TABLE 2

Formation of Hydroxylated Testosterone Metabolites (pmol/min/mg Protein) in Freshly Isolated and Cryopreserved Rat Hepatocytes in Suspension

	Freshly isolated cells				Cryopreserved cells			
Metabolite	0 min	30 min	60 min	120 min	0 min	30 min	60 min	120 min
16α-OHT	582 ± 150	522 ± 108	530 ± 89	505 ± 103	557 ± 158	523 ± 133	478 ± 104	355 ± 97
2α-OHT	523 ± 166	498 ± 150	459 ± 125	430 ± 129	421 ± 126	352 ± 110	350 ± 94	238 ± 77
6β-OHT	226 ± 34	177 ± 25	167 ± 50	128 ± 27[a]	180 ± 38	204 ± 36	135 ± 22	86 ± 18[a]
2β-OHT	60 ± 15	42 ± 18	31 ± 7[a]	17 ± 3[a]	41 ± 16	31 ± 7	22 ± 6	14 ± 4[a]
7α-OHT	57 ± 15	42 ± 10	40 ± 13	38 ± 11	36 ± 11	39 ± 10	31 ± 7	24 ± 7
15β-OHT	56 ± 12	55 ± 9	49 ± 8	50 ± 10	57 ± 7	55 ± 11	49 ± 8	41 ± 7

[a] Significantly different from the corresponding value at 0 min ($p < 0.05$; Dunnett's test).

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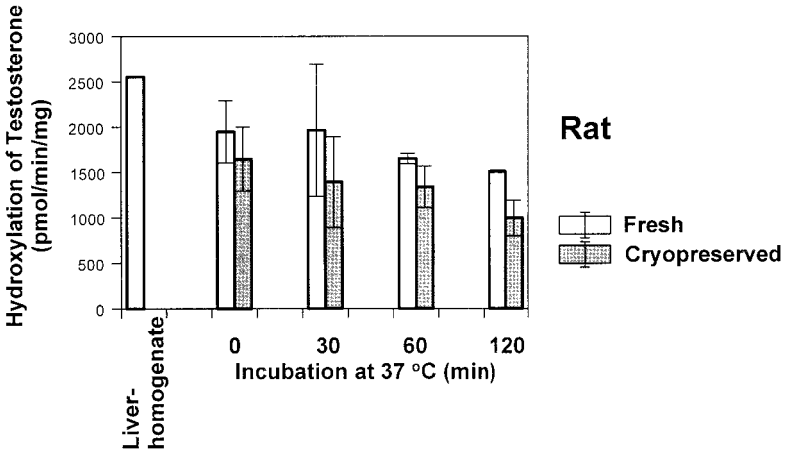

FIG. 11. Total hydroxylation of testosterone in freshly isolated and cryopreserved rat hepatocytes. The data represent mean values and standard deviations of hepatocytes obtained from four rats. (From Ref. 13.)

As for human, rat, and mouse hepatocytes, metabolic activity and viability of fresh and cryopreserved hepatocyte suspensions from male Beagle dogs was examined [20]. Albumin synthesis was unaffected by freezing (data not shown). EROD and ECOD activities were equivalent in fresh and cryopreserved dog hepatocyte suspensions over 4 h [20]. The initial EROD activities were 35 ± 11 and 23 ± 13 pmol/min/10^6 hepatocytes in cryopreserved and freshly isolated cells, respectively, but this difference was not significant. Because dog hepatocytes contain 0.95 ± 0.21 mg protein per 10^6 cells (Dr. Dietmar Utesch, personal communication), EROD activities of Beagle dogs are much higher compared to humans (Fig. 7), rats (Fig. 10), and mice (Fig. 13). When placed in suspension, EROD activities for freshly isolated and cryopreserved dog hepatocytes were ~70% after 2 h and ~55% after 4 h [20]. Initial ECOD activities of dog hepatocytes were 1080 ±

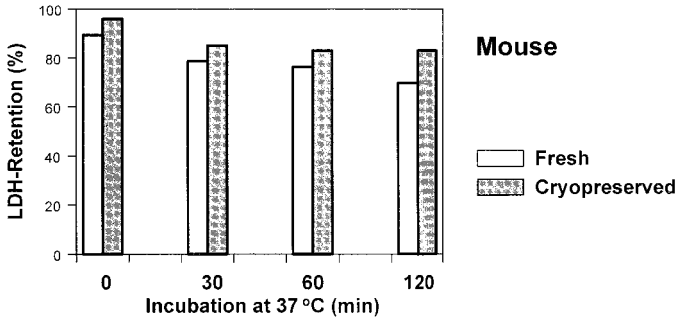

FIG. 12. Viability determined by LDH retention of cryopreserved versus freshly isolated mouse hepatocytes. The data represent mean values of hepatocytes pooled from six mice. (From Ref. 13.)

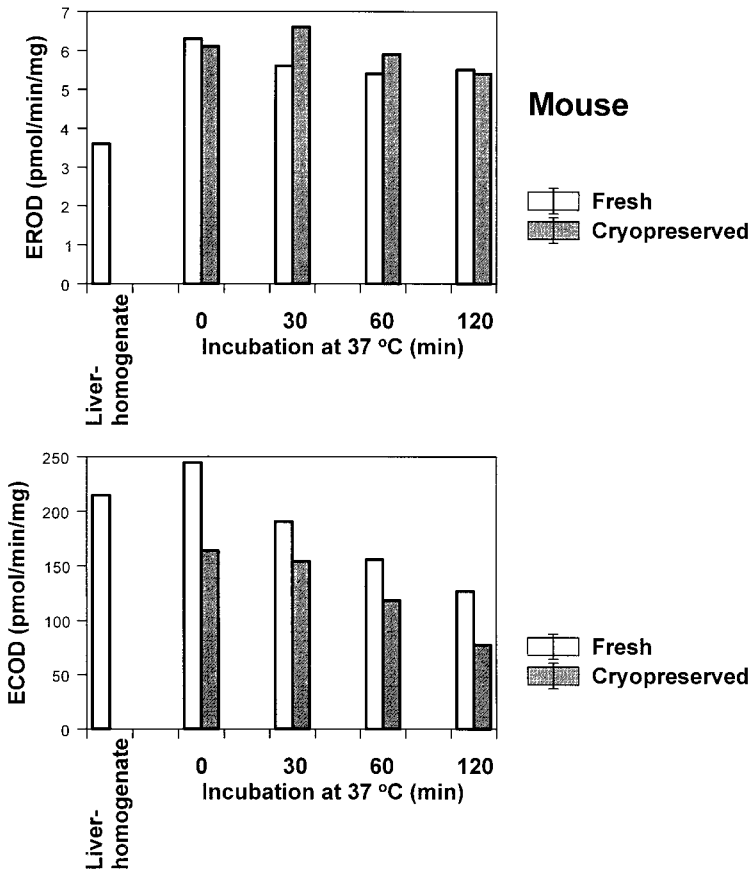

FIG. 13. EROD and ECOD activities in freshly isolated and cryopreserved mouse hepatocytes. The data represent mean values of hepatocytes pooled from six mice. (From Ref. 13.)

230 pmol/min/10^6 fresh cells and 1170 ± 337 pmol/min/10^6 cryopreserved cells [20]. Thus, as described for EROD, ECOD activities are much higher in dog hepatocytes than in hepatocytes from man (Fig. 7), rat (Fig. 10), and mouse (Fig. 13). The profile of testosterone metabolites was unaffected by freezing. The main testosterone metabolites formed by Beagle dog hepatocytes (cryopreserved as well as freshly isolated) are 6β-, 16α-, 16β-, and 2β-OHT. The total testosterone hydroxylation activity was 815 ± 33 pmol/min/10^6 cells in freshly isolated hepatocytes and 463 ± 24 pmol/min/10^6 cells in cryopreserved hepatocytes [20]. However, almost identical activities were obtained for freshly isolated and cryopreserved dog hepatocytes after supplementation with 250 μ*M* NADPH. Thus, the loss in activity was due to leakage or oxidation of NADPH. When cryopreserved dog hepatocytes were placed in suspension, the total testosterone hydroxylation declined to 63 ± 6% of the initial activity after 4 h.

In conclusion, viability and phase I metabolism of cryopreserved hepatocytes held in suspension for up to 2 h are in a range that is acceptable for metabolic studies.

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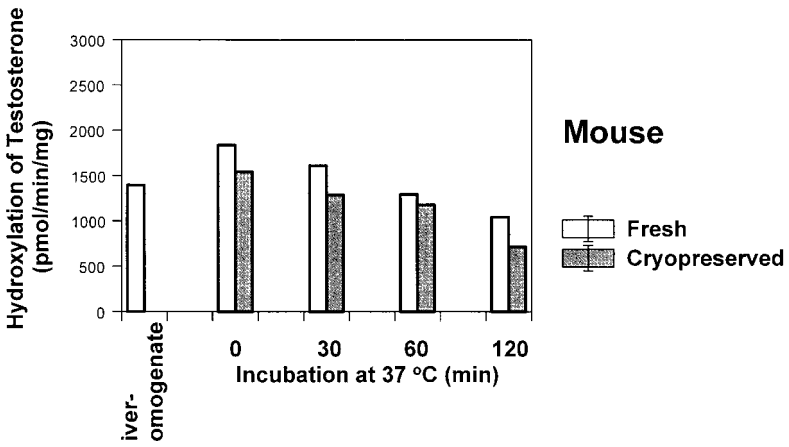

FIG. 14. Total hydroxylation of testosterone in freshly isolated and cryopreserved mouse hepatocytes. The data represent mean values of hepatocytes pooled from six mice. (From Ref. 13.)

VI. CRYOPRESERVED HEPATOCYTES AS A METABOLIZING SYSTEM IN MUTAGENICITY TESTS

In the next experiments, a more complex criterion was investigated: Intact rat hepatocytes were used as a metabolizing system in the Ames test. 2-Aminoanthracene (2-AA) is an aromatic amine that is activated by CYP1A2, converting it to the hydroxylamine, which is further activated by sulfonation and O-acetylation. 2-Aminoanthracene was activated to the same extent by freshly isolated and cryopreserved hepatocytes (Fig. 15 [9]). However, for the other substances, benzo[*a*]pyrene, dimethylbenz[*a*]anthracene, and cyclophosphamide activation by cryopreserved hepatocytes was weaker (Fig. 15). To discover the reason, we next concentrated on benzo[*a*]pyrene metabolism.

V. LOSS OF THE COFACTOR NADPH IN CRYOPRESERVED HEPATOCYTES

The total amount of benzo[*a*]pyrene metabolites formed by 1×10^6 cryopreserved or freshly isolated rat hepatocytes per milliliter of incubation buffer in 90 min was determined (Fig. 16 [10]). A significant decrease in benzo[*a*]pyrene metabolism of cryopreserved versus freshly isolated hepatocytes was observed. Thus, the data fit to the abovementioned results of the Ames test, where cryopreserved hepatocytes were less efficient in activating benzo[*a*]pyrene to a mutagen (Fig. 15). Hepatocytes shock frozen in liquid nitrogen were almost inactive (Fig. 16).

The decrease in metabolism of cryopreserved hepatocytes could be due to two mechanisms: inactivation of cytochrome P-450 enzymes or loss of the cofactor NADPH due to cell membrane damage. The second explanation seems to be true, because the addition of an NADPH generating system to the incubation mixture increased benzo[*a*]pyrene me-

TABLE 3

Formation of Hydroxylated Testosterone Metabolites (pmol/min/mg Protein) in Freshly Isolated and Cryopreserved Mouse Hepatocytes in Suspension

	Freshly isolated cells				Cryopreserved cells			
Metabolite	0 min	30 min	60 min	120 min	0 min	30 min	60 min	120 min
6β-OHT	136 ± 84	120 ± 87	96 ± 77	69 ± 67	137 ± 70	101 ± 60	96 ± 45	60 ± 35
15α-OHT	61 ± 47	50 ± 45	45 ± 41	41 ± 36	53 ± 36	51 ± 33	52 ± 35	46 ± 44
6α-OHT	58 ± 27	58 ± 34	54 ± 31	39 ± 27	50 ± 16	45 ± 12	46 ± 15	33 ± 17
16α-OHT	42 ± 40	38 ± 36	26 ± 20	23 ± 20	42 ± 31	29 ± 25	31 ± 23	22 ± 19
7α-OHT	42 ± 36	40 ± 39	56 ± 47	50 ± 48	48 ± 44	36 ± 33	33 ± 31	25 ± 20
2α-OHT	38 ± 23	33 ± 27	36 ± 25	30 ± 25	35 ± 7	42 ± 27	42 ± 30	34 ± 17
2β-OHT	25 ± 5	22 ± 9	17 ± 12	16 ± 6	27 ± 16	30 ± 8	40 ± 24	17 ± 11

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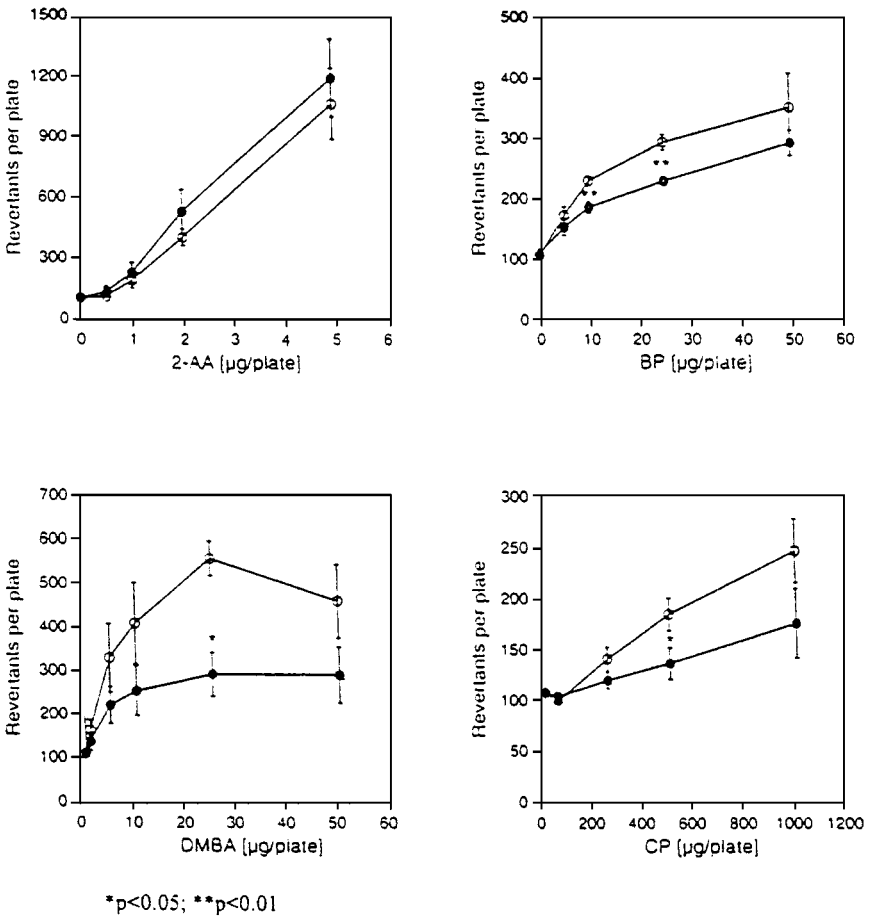

FIG. 15. Freshly isolated (○) and cryopreserved (●) rat hepatocytes as metabolizing systems in the Ames test with *Salmonella typhimurium* TA 100 using 2-aminoanthracene (2-AA), benzo[*a*]pyrene (BP), 7,12-dimethylbenz[*a*]anthracene (DMBA), and cyclophosphamide (CP) as test substances. (From Ref. 9.)

tabolite formation by cryopreserved cells to approximately the level of freshly isolated cells (Fig. 16). When cryopreserved hepatocytes were purified by Percoll centrifugation after thawing (a technique that removes cells with damaged membranes), benzo[*a*]pyrene metabolism was equivalent to that for freshly isolated cells (Fig. 16). At a first glance, it may seem contradictory that phase I metabolism in cryopreserved hepatocytes (without Percoll centrifuation) was only minimally altered (Section III), but benzo[*a*]pyrene metabolism was reduced. However, it should be taken into account that the experiments on phase I metabolism (Section III) were performed with NADPH-fortified homogenate, in contrast to the experiments on benzo[*a*]pyrene metabolism where intact hepatocytes were used. Thus, a loss of the cofactor will influence experiments with intact hepatocytes, but not the assays with NADPH-fortified hepatocyte homogenate. To avoid problems due to the loss of cofactor, Percoll centrifugation should be performed routinely after thawing of cryopreserved hepatocytes.

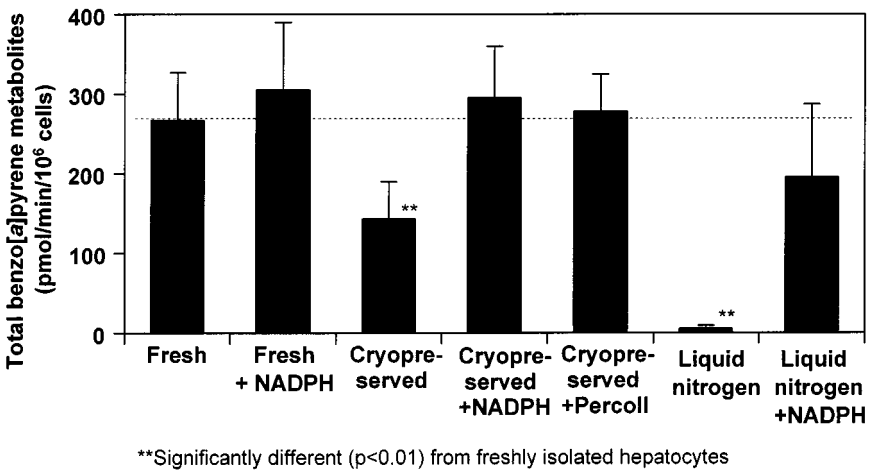

FIG. 16. Influence of Percoll centrifugation and addition of an NADPH generating system (2 m*M* NADP-sodium and 2.5 m*M* glucose-6-phosphate) on benzo[*a*]pyrene metabolism of rat hepatocytes. (From Ref. 10.)

VI. PHASE II METABOLISM OF CRYOPRESERVED HEPATOCYTES

Next, we examined activities of phase II metabolizing enzymes in cryopreserved hepatocytes, in a first step without Percoll purification [13]. Activity of UDP-glucuronosyltransferase (UDP-GT) was determined using two substrates: 4-hydroxybiphenyl (HOBI) and 4-methylumbelliferone (MUF). HOBI is metabolized by phenobarbital-inducible UDP-GT isoenzymes, whereas MUF is a substrate for the 3-methylcholanthrene-inducible UDP-GT isoforms [21,22]. Using HOBI as a substrate, UDP-GT seems to be almost unaltered by the cryopreservation procedure (Fig. 17 [13]), showing typical interspecies differences with highest activities for mice, lowest for man, and the rat being intermediate. Similar results were obtained with MUF as a substrate (Fig. 18).

However, glutathione S-transferase (GST) activity determined with 1-cloro-2,4-dinitrobenzene as a substrate is strongly reduced in cryopreserved human hepatocytes in contrast to cells from rats and mice, where GST activity is well maintained (Fig. 19). A similar problem exists with sulfotransferase activity (substrate: 2-naphthol) for cryopreserved hepatocytes of the rat (Fig. 20). The reason for these interspecies differences in the decrease of different enzymes is not known. It can be speculated that the cofactor system is disturbed. However, these problems could be overcome by Percoll centrifugation. The GST activity of cryopreserved human hepatocytes and similarly sulfotransferase activity of rat hepatocytes were improved by Percoll centrifugation, not completely to the level of freshly isolated cells but to an extent that should be acceptable for metabolic studies (Table 4 [10]). For microsomal and soluble epoxide hydrolase (mEH, sEH), similar activities for freshly isolated and cryopreserved hepatocytes were obtained even without Percoll centrifugation (Table 4).

In conclusion, Percoll centrifugation performed after thawing of cryopreserved cells guarantees acceptable activities, even of the more problematic enzymes such as GST and

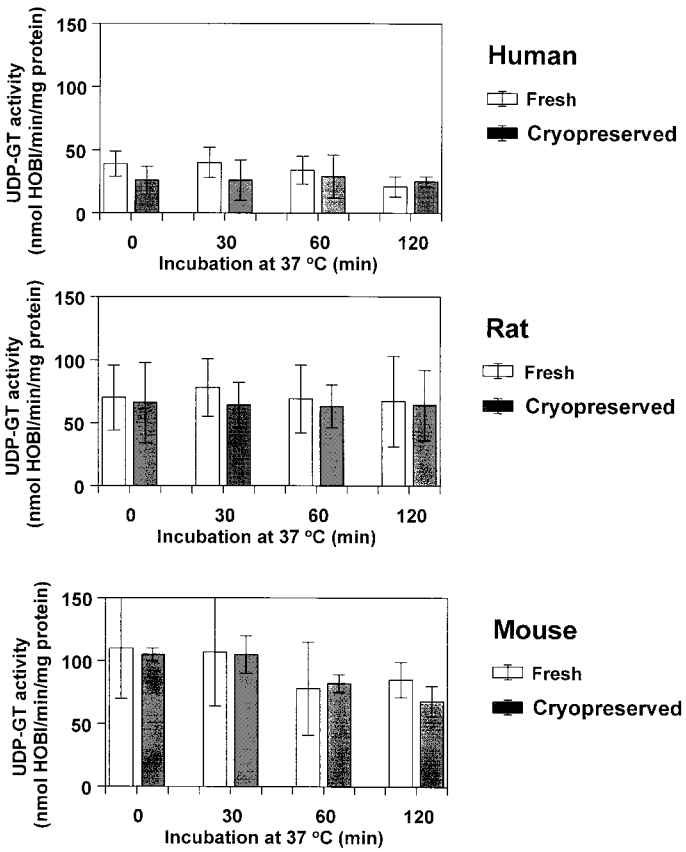

FIG. 17. UDP-glucuronosyltransferase activity using 4-hydroxybiphenyl as a substrate of freshly isolated and cryopreserved hepatocytes of human, rat, and mouse. Data are expressed as mean ± standard deviation of seven independent human and four independent rat hepatocyte isolations and their corresponding cryopreserved counterparts. In the case of mouse hepatocytes, cells from three to four mice were pooled and the results are expressed as the mean ± standard deviation of four such pools. (From Ref. 13.)

sulfotransferase. Thus, Percoll centrifugation was included in all further experiments with intact hepatocytes.

VII. COMPLEX METABOLISM BY CRYOPRESERVED AND FRESHLY ISOLATED HEPATOCYTES: PHASE I AND PHASE II METABOLISM OF BENZO[*a*]PYRENE

Benzo[*a*]pyrene is transformed to numerous metabolites as the result of a complex interaction of phase I and phase II enzymes. A technique using ^{14}C-labeled benzo[*a*]pyrene combining several extractive and chromatographic steps allows direct analysis of these

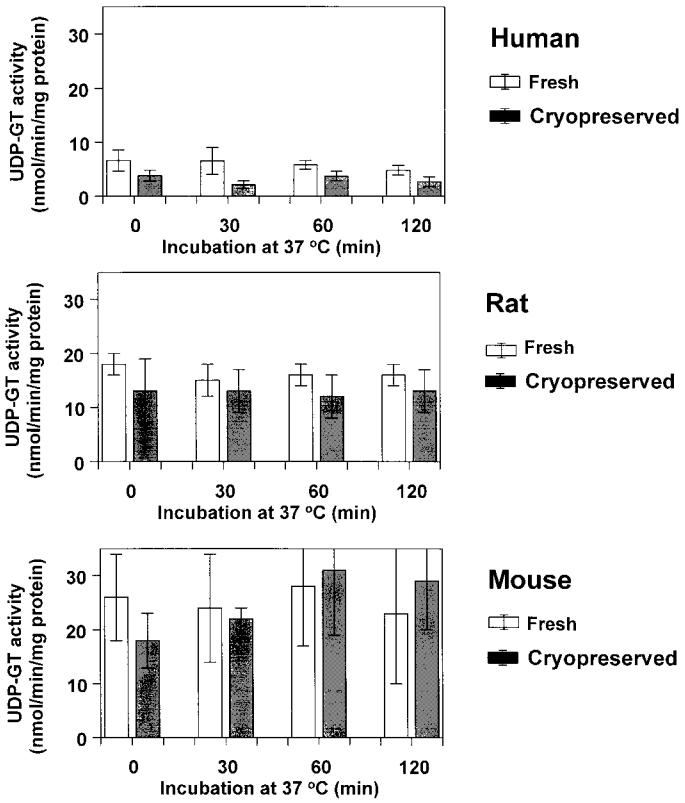

FIG. 18. UDP-glucuronosyltransferase activity using 4-methylumbelliferone as a substrate of freshly isolated and cryopreserved hepatocytes of human, rat, and mouse. Data are expressed as the mean ± standard deviation of seven independent human and four independent rat hepatocyte isolations and their corresponding cryopreserved counterparts. In the case of mouse hepatocytes, cells from three to four mice were pooled and the results are expressed as the mean ± standard deviation of four such pools. (From Ref. 13.)

metabolites [23]. Phase I metabolites and sulfates can be extracted by an ethyl acetate aceton mixture; the glucuronides from the remaining aqueous phase by acidification and extraction with ethyl acetate and the glutathione conjugates represent the remaining fraction after precipitation of proteins with methanol.

Cryopreserved as well as freshly isolated intact rat hepatocytes were incubated with 80 μ*M* benzo[*a*]pyrene in suspension for 90 min. Cryopreservation was performed by the optimized technique, including Percoll centrifugation. Very similar patterns of metabolites were produced by freshly isolated and cryopreserved hepatocytes (Fig. 21 [23]). Not a single metabolite was lost due to the cryopreservation procedure. Quantification of the single groups of metabolites (phase I metabolites and sulfates, glucuronides, and glutathione conjugates) results in very similar amounts produced by freshly isolated and cryopreserved cells (Fig. 22).

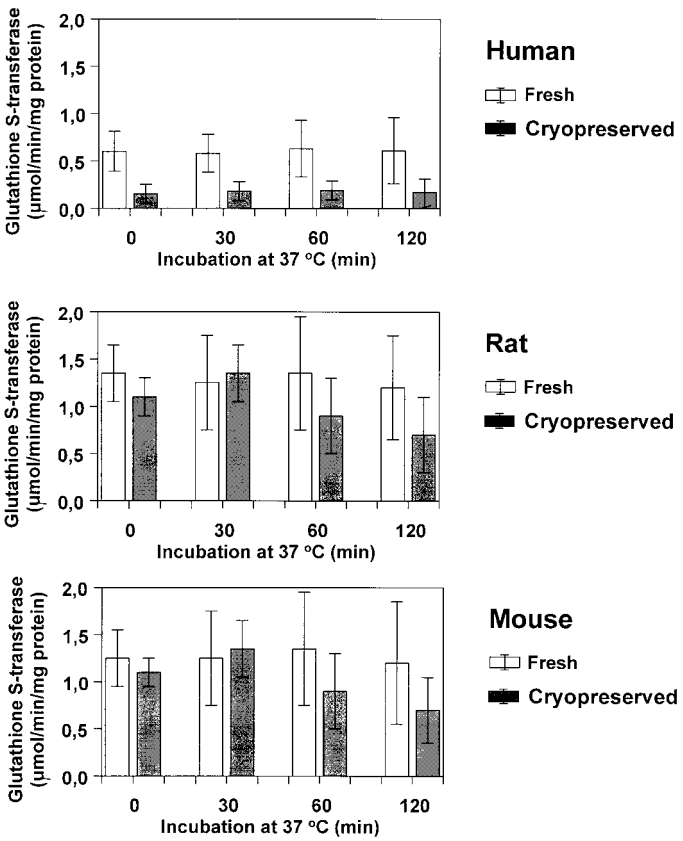

FIG. 19. Glutathione S-transferase activity using 1-chloro-2,4-dinitrobenzene as a substrate of freshly isolated and cryopreserved hepatocytes of human, rat, and mouse. Data are expressed as the mean ± standard deviation of seven independent human and four independent rat hepatocyte isolations and their corresponding cryopreserved counterparts. In the case of mouse hepatocytes, cells from three to four mice were pooled and the results are expressed as the mean ± standard deviation of four such pools. (From Ref. 13.)

VIII. CULTURES WITH CRYOPRESERVED HEPATOCYTES: ENZYME INDUCTION

All experiments presented so far concerned short-term applications of hepatocytes in suspension. Hepatocytes in suspension have the advantage to metabolize similar to hepatocytes in vivo, but the disadvantage to have a time limit of about 2–4 h. After longer incubation periods, some enzyme activities decrease markedly. Thus, cryopreserved hepatocytes in suspension can be applied for short-term metabolic studies or as metabolizing systems in mutagenicity assays, but not to study enzyme induction. For this purpose, hepatocyte cultures are required. The use of hepatocyte cultures is now well established for the study of drug–drug interactions and several favorable reports of the correlation of

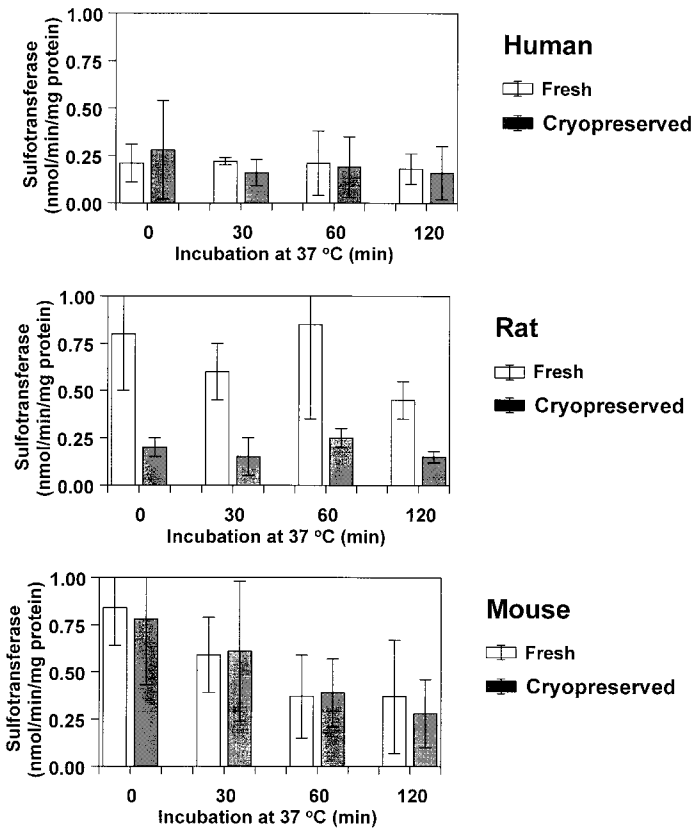

FIG. 20. Sulfotransferase activity using 2-naphthol as a substrate of freshly isolated and cryopreserved hepatocytes of human, rat, and mouse. Data are expressed as the mean ± standard deviation of seven independent human and four independent rat hepatocyte isolations and their corresponding cryopreserved counterparts. In the case of mouse hepatocytes, cells from three to four mice were pooled and the results are expressed as the mean ± standard deviation of four such pools. (From Ref. 13.)

such in vitro data with those obtained in vivo have been published [2–4]. However, the major obstacle to the use of hepatocyte cultures is that freshly isolated hepatocytes are required. Freshly isolated human hepatocytes represent the most relevant model for in vitro identification of drug–drug interactions. Because human liver tissue and, consequently, human hepatocytes are only occasionally available and only a few laboratories have established procedures for the isolation of highly viable and functional hepatocytes, human hepatocyte cultures are not used routinely in drug development. Thus, a future goal should be to establish hepatocyte cultures with cryopreserved hepatocytes. However, a recent consensus of an expert panel was that, in contrast to short-term metabolism and toxicity studies with hepatocytes in suspension, the use of hepatocytes cultured after cryopreservation to study enzyme induction is still problematic [15]. In general, only a small percentage (less than 50%) of the cryopreserved hepatocytes can attach onto collagen-coated plates [5]. Only limited success has been achieved with the induction of CYP1A2

TABLE 4

Influence of Percoll Centrifugation on Cryopreserved Hepatocytes

	Freshly isolated	Cryopreserved	Cryopreserved + Percoll
		Rat	
Sulfotransferase (nmol/min/mg)	2.52 ± 0.25	1.07 ± 0.32	2.06 ± 0.69
UDP-GT (nmol/min/mg)	16.1 ± 3.0	13.5 ± 1.5	20.4 ± 2.4
mEH (nmol/min/mg)	1.62 ± 0.31	1.37 ± 0.37	nt[a]
sEH (nmol/min/mg)	1.52 ± 4.4	1.62 ± 5.2	nt
		Human	
GST (μmol/min/mg)	0.41 ± 0.05	0.13 ± 0.02	0.35 ± 0.06

[a] nt: Not tested.

and CYP3A4 isoforms in hepatocytes cultured after cryopreservation, but the enzyme activity is much lower than that for freshly isolated cells [5]. Thus, an expert panel recommended that enzyme-induction studies should continue to be performed with freshly isolated hepatocytes unless further progress can be achieved [5].

It would be an enormous practical progress if cryopreserved hepatocyte cultures of all species of interest would be available (i.e., that they could be taken out of the freezer when needed). Therefore, we applied an optimized technique to cryopreserve hepatocytes in suspension [10,13] and cocultured the thawed hepatcytes with the nonparenchymal epithelial rat liver cell line NEC1 on collagen-coated dishes [16,24,25]. In previous studies, we have shown that NEC1 cells stablize differentiated functions in rat liver parenchymal cells [25a].

Because previous studies reported an improvement of differentiated functions by preincubation of hepatocytes in a carbogen-equilibrated buffer with high D-glucose concentration (~25 m*M*) at 37°C immediately before cryopreservation [15], we compared data obtained by the latter technique (''warm'' technique) with results using the ''cold'' isolation technique that did not include carbogen equilibration and all centrifugation and resuspension steps were performed at 4°C [16,24,25]. A clear improvement in attachment efficiency was obtained using the ''warm'' technique (Fig. 23). In addition, the number of viable hepatocytes per rat was 818 ± 190 million for the ''warm'' technique compared to only 401 ± 129 million for the ''cold'' technique. As expected, Percoll centrifugation clearly improved attachment efficiency and Trypan Blue exclusion after cryopreservation (Fig. 23). Surprisingly, the Trypan Blue exclusion rate determined after cryopreservation of hepatocytes isolated by the ''warm'' technique was slightly lower when compared to the ''cold'' technique (Fig. 23). However, this difference might be due to toxicity of DMSO, because stepwise addition of DMSO was performed at 4°C for the ''cold'' technique but at room temperature for the ''warm'' technique. Thus, in our recent experiments, hepatocytes isolated by the ''warm'' technique were cooled down to 4°C immediately after preincubation in carbogen-equilibrated suspension buffer before the addition of DMSO, resulting in a similar Trypan Blue exclusion rate as for hepatocytes isolated by the ''cold'' technique. However, the extent to which cooling before DMSO addition improved attachment efficiency was only marginal (data not shown).

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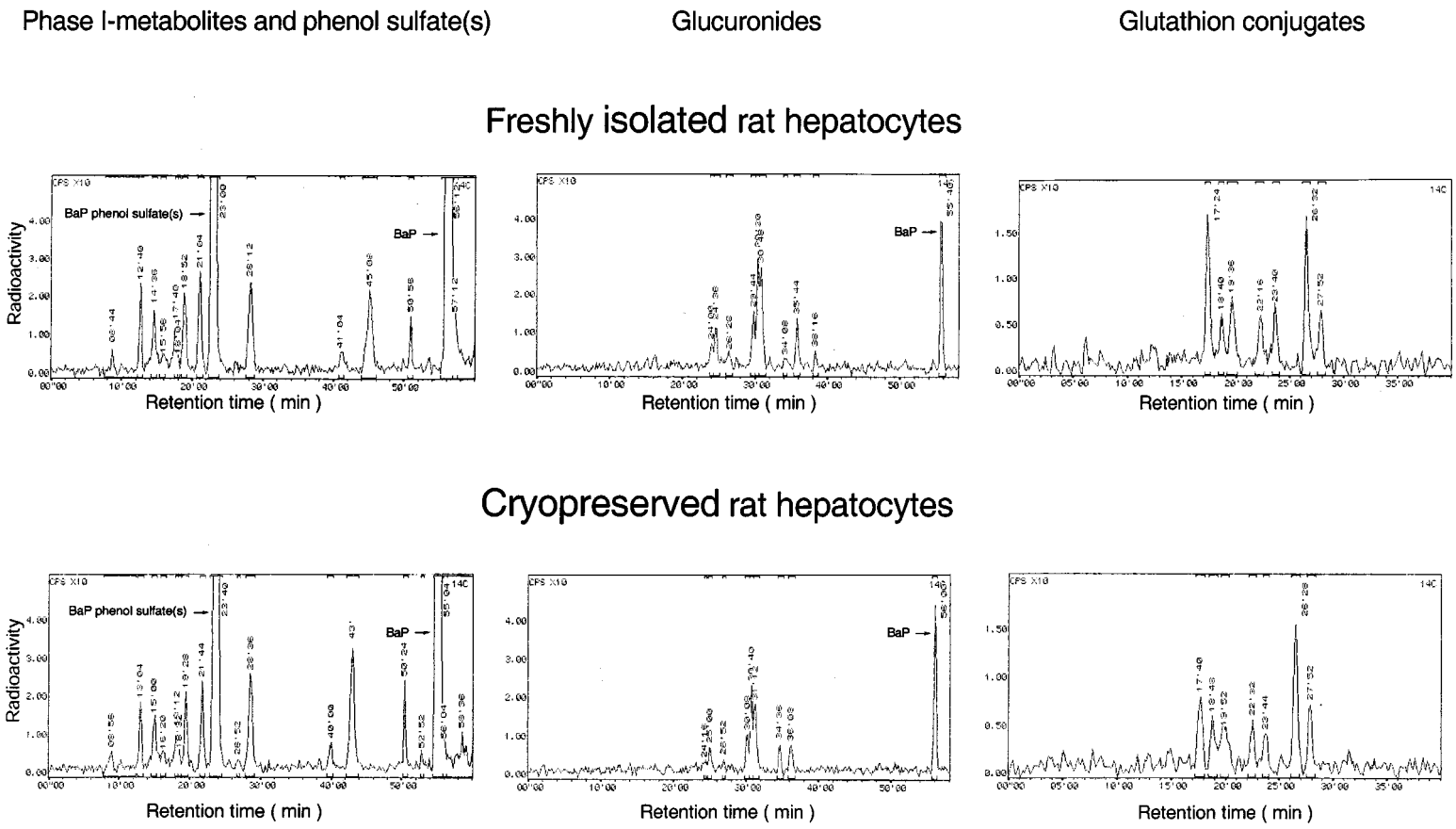

FIG. 21. Phase I and phase II metabolites of benzo[*a*]pyrene formed by freshly isolated and cryopreserved rat hepatocytes. (From Ref. 23.)

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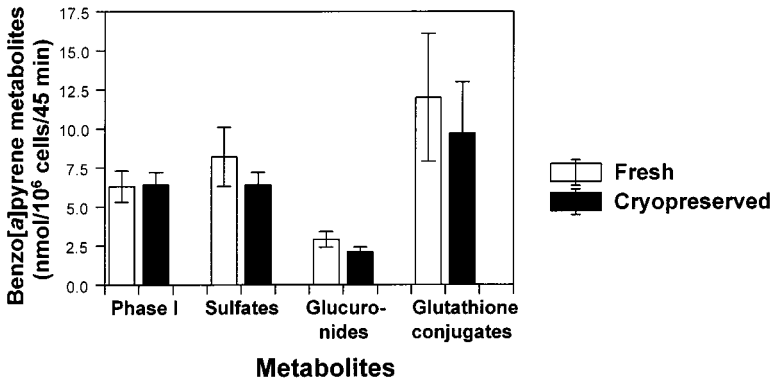

FIG. 22. Quantification of phase I and phase II metabolites of benzo[*a*]pyrene formed by freshly isolated and cryopreserved rat hepatocytes. (From Ref. 23.)

In cocultures with freshly isolated and cryopreserved hepatocytes, EROD and PROD activities were determined after incubation with the standard inducers 3-methylcholanthrene (3-MC) and phenobarbital (PB) (Fig. 24). EROD and PROD activities in the solvent controls were close to the detection limit (<3 pmol/min/min). Thus, we did not determine induction factors, because the latter would be strongly influenced by experimental variation of the very small basal activities. It seems to be more reasonable to compare the absolute activities after induction: Incubation with 3-MC resulted in EROD activities of 95 and 59 pmol/min/mg for freshly isolated and cryopreserved cultures, respectively (results for hepatocytes obtained by the ''warm'' technique). The corresponding 7-pentoxyresorufin O-dependentylation (PROD) activities after induction with PB were 40 and 15 pmol/mg/min, respectively (Fig. 24). Thus, the levels of the induced enzyme activity in the cryopreserved cultures is reduced to 62% for 3-MC (EROD activity) and 38% for PB (PROD activity) as compared with the values in fresh cultures.

No significant difference in enzyme induction was observed for cultures prepared with hepatocytes isolated by the ''cold'' and ''warm'' isolation tecchniques. Obviously, the ''warm'' technique improves cell harvest and attachment efficiency, but after attachment, enzyme induction is not affected by the isolation technique.

Induction of the CYP2B-dependent PROD by PB in our system is stronger when compared to previously published data. For instance, Wortelboer et al. [26] reported a significant induction of PROD activity by phenobarbital with 5.4 ± 1.7 and 11.1 ± 2.5 pmol/min/mg for controls and hepatocyte cultures incubated with 1.5 m*M* PB for 72 h respectively. Thus, the cryopreserved cultures in the present study are at least as active (15 pmol/min/mg after induction with PB) as other previously published culture systems using freshly isolated hepatocytes. The activity of our system is rather underestimated because we used hepatocyte homogenate, whereas most published studies prepared microsomes. (Although microsomes exhibit about fivefold higher cytochrome P-450 activities per milligram of protein than hepatocyte homogenate, we prefer the use of homogenate for determination of enzyme activities when optimizing culture and cryopreservation techniques; this saves work and avoids unnecessary procedures that may cause experimental variation. The stronger PROD induction in the present study might be due to coculturing hepatocytes with the nonparenchymal epithelial rat liver cell line NEC1, whereas most

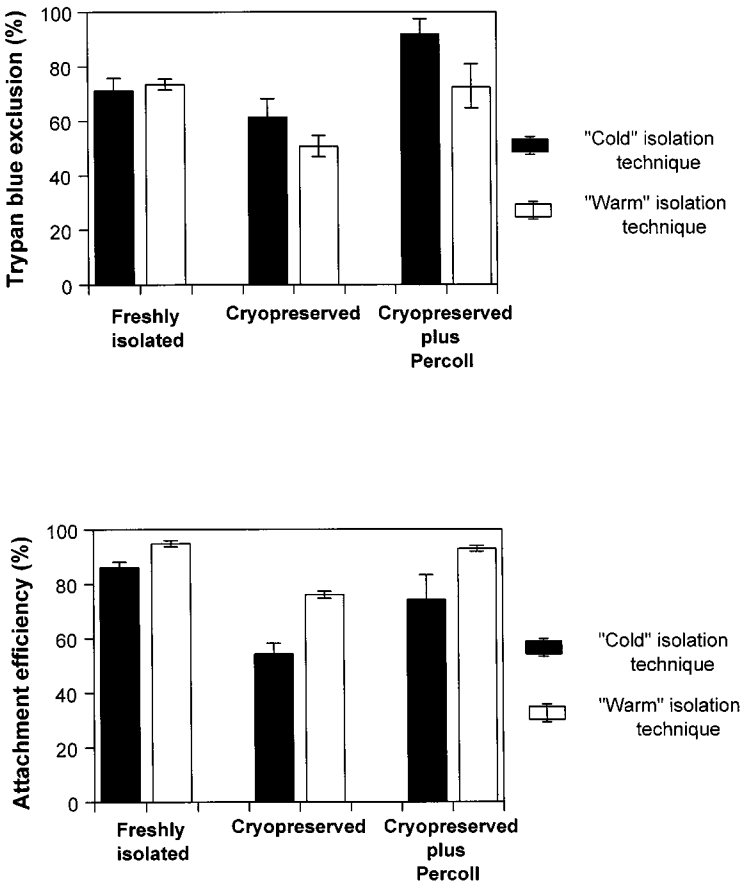

FIG. 23. Trypan Blue exclusion rate and attachment efficiency of freshly isolated, cryopreserved, and cryopreserved plus Percoll-purified rat hepatocytes. Results obtained with the ''warm'' isolation technique (including 30 min preincubation at 37°C in a carbogen-equilibrated buffer) were compared to data obtained with the ''cold'' isolation technique (without preincubation, all manipulations after collagenase digestion performed at 4°C). The data are mean values ± standard deviations of three independent experiments. (From Ref. 16.)

previous studies applied hepatocyte monocultures [26]. Although our cryopreserved cultures compare well to other in vitro systems, it should be considered that induction of PROD activity by PB in vivo is higher [26]. For instance, application of a 0.1% solution of PB in drinking water to male Wistar rats induced microsomal PROD activity to 322 ± 109 pmol/min/mg microsomal protein (controls: 8 ± 1 pmol/min/mg) [26].

O-Deethylation of 7-ethoxyresorufin (EROD) is a reaction catalyzed by cytochrome P-450 1A1 and 1A2 [17]. As in the case of PROD, induction of EROD activity in vivo was stronger than in cryopreserved hepatocyte cultures in vitro: injection (i.p.) of 3-methylcholanthrene (27 mg/kg/day) to male Sprague–Dawley rats for 4 days resulted in an EROD activity of 387 pmol/min/mg microsomal protein [27]. This is more than threefold

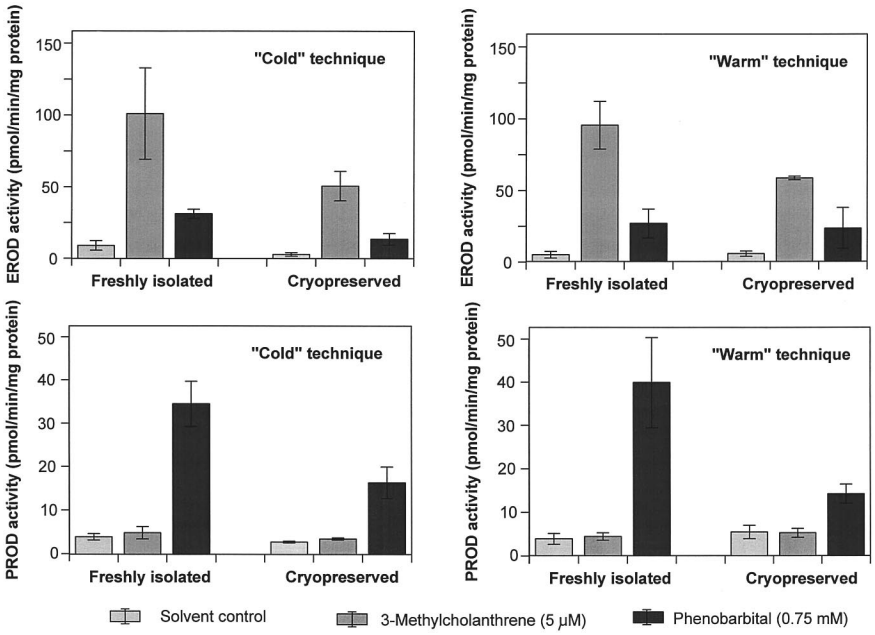

FIG. 24. Induction of EROD and PROD activity by 3-methylcholanthrene (5 μ*M*) and phenobarbital (0.75 m*M*) in rat hepatocyte cocultures prepared with freshly isolated and cryopreserved hepatocytes. In addition, the influence of different isolation procedures, the ''cold'' and ''warm'' isolation techniques, was examined. The data are mean values ± standard deviations of three or four independent experiments. (From Ref. 16.)

higher compared to cryopreserved hepatocyte cultures incubated with 5 μ*M* 3-MC for 3 days in the present study, but the difference may be overestimated because the latter activity was determined in cell homogenate.

Hydroxylation of testosterone by cocultures with cryopreserved and freshly isolated hepatocytes were similar if induction factors were compared (Fig. 25; Tables 5 and 6). In addition, the patterns of testosterone hydroxylation products induced by PB and 3-MC are similar to published in vivo studies with male Sprague–Dawley rats (Tables 5 and 6). Although there are discrepancies among the above-mentioned in vivo studies [26–29] (Tables 5 and 6), PB mainly affects 16β-OHT formation and 16β-OHT in rats. These activities also show the highest induction factors for the cryopreserved as well as the freshly isolated cocultures. Compared to 16β-OHT, smaller but still significant induction factors were observed for 2β-, 6α-, 6β-, 7α-, 15β-, and 16α-OHT in in vivo studies (Table 3) [27–29]. With the exception of 6α-OHT formation, all other mentioned hydroxylated testosterone metabolites were also induced in cryopreserved cultures in vitro. It should be noted that the data obtained from the three in vivo studies are controversial for single metabolites and that 6α-OHT was determined only in one of the three studies. All hydroxylated testosterone metabolites that were significantly induced by PB in a previous study [26] using fresh rat hepatocyte monocultures (6β-, 7α-, 15β-, 16α-, and 16β-OHT) were also significantly induced in the cryopreserved cultures in the present study (Table 5).

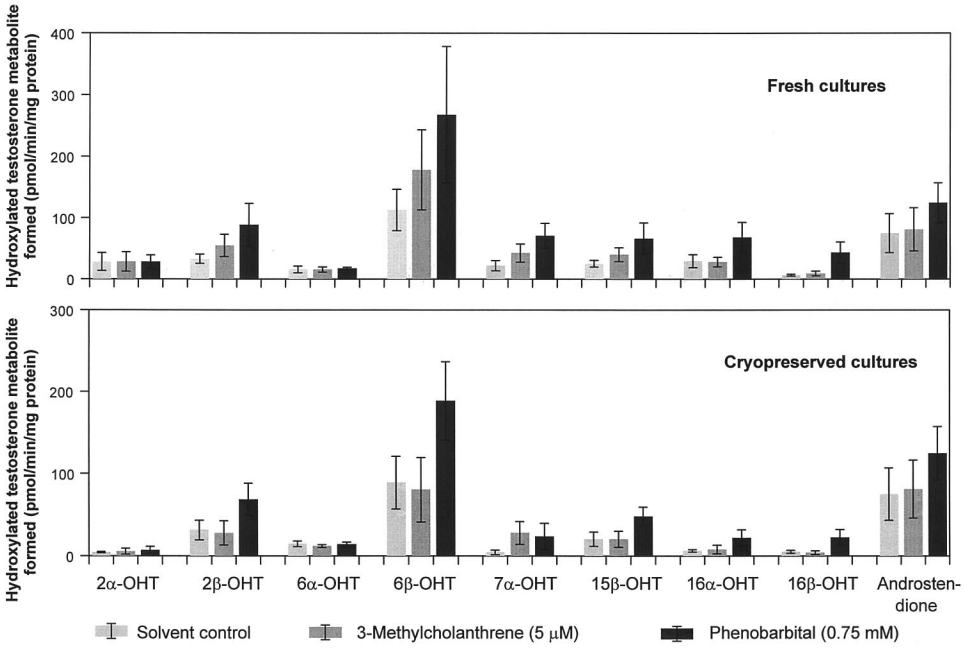

FIG. 25. Regioselective and stereoselective formation of hydroxylated testosterone metabolites (OHT) by hepatocyte cocultures prepared with freshly isolated (upper panel) and cryopreserved hepatocytes (lower panel). Hepatocyte cocultures were incubated with solvent, 5 μ*M* 3-methylcholanthrene or 0.75 m*M* phenobarbital for 72 h. Data are mean values ± standard deviations of four or five independent experiments. (From Ref. 16.)

As for PB, a good agreement between in vivo data and the results obtained by cryopreserved hepatocyte cultures in vitro was observed after induction with 3-MC (Table 6). A clear induction in all three in vivo studies [27–29] was solely observed for 7α-OHT, with induction factors ranging from 2.8 to 3.7 (Table 6). Similarly, 7α-OHT was the only testosterone hydroxylation product induced by 3-MC in vitro in cocultures, resulting in induction factors of 2.0 and 6.6 for freshly isolated and cryopreserved cultures, respectively. Concerning the induction of 7α-OHT, cryopreserved cultures at a first glance seem to be superior to fresh cultures with induction factors of 6.6 (cryopreserved) and 2.0 (fresh). However, this interpretation should be treated with caution, as the increase in the induction factor is mainly due to a decrease in 7α-OHT activity for the cryopreserved controls (and not an increase in the 3-MC treated cells) (Fig. 25). This leads to the main disadvantage of the cryopreserved hepatocyte cocultures [i.e., reduction of the absolute testosterone hydroxylation activities (Fig. 25)] although induction factors are well maintained (Tables 5 and 6). For instance, 6β-OHT, the main metabolite in solvent controls, was reduced to 79% in cryopreserved cultures. Furthermore, in cryopreserved cultures, 7α- and 16β-hydroxylation activities, which are the main activities induced by 3-MC and PB, respectively, were reduced to 66% and 52% of the activities in freshly isolated hepatocyte cultures. A second disadvantage is a relatively high loss of cells due to cryopreservation, thawing, and Percoll centrifugation. Thus, only about 20% of the originally isolated

TABLE 5

Induction Factors of OHT After Treatment of Rats In Vivo (Literature Data) and After Incubation of Cocultures of Freshly Isolated or Cryopreserved Rat Hepatocytes with Phenobarbital

Study	Rat strain; in vivo/in vitro	Treatment	Induction factors								4-Androsten-3,17-dione
			2α-OHT	2β-OHT	6α-OHT	6β-OHT	7α-OHT	15β-OHT	16α-OHT	16β-OHT	
			Treatment in vivo								
Chung et al. [29]	Male Sprague–Dawley; in vivo	80 mg/kg/day; i.p.; for 4 days	0.1	4.2	nt[a]	3.4	0.9	nt	0.5	14.0	0.7
Parkinson et al. [27]	Male Sprague–Dawley; in vivo	85 mg/kg/day; in diet; for 2 weeks	0.2	4.0	4.2	3.1	4.4	3.9	1.1	21.8	1.5
Sohn et al. [28]	Male Sprague–Dawley; in vivo	80 mg/kg/day; i.p.; for 3 days	0.4	nt	nt	1.3	1.4	nt	2.7	>30[b]	nt
Wortelboer et al. [26]	Male Wistar; in vivo	0.1% in drinking water for 5 days	0.5	nt	nt	2.8	2.2	3.6	1.7	67.1	2.2
			Treatment of freshly isolated hepatocytes in culture								
Wortelboer et al. [26]	Male Wistar; in vitro	Hepatocyte monoculture; 1.5 m*M* for 3 days	0.7	nt	nt	6.0	2.4	7.3	0.9	>7[c]	1.1
			Treatment of cryopreserved versus freshly isolated hepatocytes in culture								
Hengstler et al. [16]	Male Sprague–Dawley; in vitro	Hepatocyte coculture; 0.75 m*M* for 3 days									
		''Warm'' technique									
		a. Freshly isolated	1.0	2.7	1.1	2.5	3.2	2.6	2.3	7.0	1.7
		b. Cryopreserved	1.6	2.2	1.0	2.1	2.9	2.4	3.6	4.5	0.9
		''Cold'' technique									
		a. Freshly isolated	1.8	2.7	1.1	2.4	3.4	2.1	2.1	4.2	1.4
		b. Cryopreserved	1.2	1.5	0.9	1.5	3.7	1.4	2.2	5.1	1.2

[a] nt: Not tested.
[b] 16β-OHT increased from not detectable to 0.30 nmol/min/mg protein.
[c] 16β-OHT increased from <2 to 13.9 nmol/min/mg protein.

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TABLE 6

Induction Factors of OHT After Treatment of Rats In Vivo (Literature Data) and After Incubation of Cocultures of Freshly Isolated or Cryopreserved Rat Hepatocytes with 3-Methylcholanthrene

Study	Rat strain; in vivo/in vitro	Treatment	Induction factors								4-Androsten-3,17-dione
			2α-OHT	2β-OHT	6α-OHT	6β-OHT	7α-OHT	15β-OHT	16α-OHT	16β-OHT	
			Treatment in vivo								
Chung et al. [29]	Male Sprague–Dawley; in vivo	25 mg/kg/day; i.p.; for 3 days	0.2	2.3	nt[a]	1.4	3.7	nt	0.3	1.2	1.2
Parkinson et al. [27]	Male Sprague–Dawley; in vivo	27 mg/kg/day; i.p.; for 4 days	0.4	1.2	1.9	1.4	2.8	1.3	0.5	0.7	0.6
Sohn et al. [28]	Male Sprague–Dawley; in vivo	20 mg/kg/day; i.p.; for 3 days	1.0	nt	nt	1.5	3.2	nt	n.d.[b]	nt	nt
			Treatment of cryopreserved versus freshly isolated hepatocytes in culture								
Hengstler et al. [16]	Male Sprague–Dawley; in vitro	Hepatocyte coculture; 5 μ*M* for 3 days									
		''Warm'' technique									
		a. Freshly isolated	1.0	1.7	1.0	1.6	2.0	1.6	1.0	1.5	1.1
		b. Cryopreserved	1.3	0.9	0.8	0.9	6.6	1.0	1.3	0.8	0.6
		''Cold'' technique									
		a. Freshly isolated	0.8	0.7	1.0	0.7	2.0	1.7	1.0	0.7	0.9
		b. Cryopreserved	0.6	0.9	1.0	1.0	6.1	0.8	0.8	0.8	1.9

[a] nt: Not tested.
[b] Not detectable in 3-methylchloanthrene-treated and untreated animals.

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TABLE 7

Standard Operation Procedure for Isolation of Human Hepatocytes

- Human liver samples can be obtained from patients that undergo liver resection due to liver metastasis.
- The resected tissue should be immediately transferred into ice-cold suspension buffer (composition: see Table 8).
- The resected liver tissue can be stored in ice-cold suspension buffer for at least 4 h. The ischemia phase *in situ* (before resection of the tissue) was ≤30 min for all tissue specimens we have examined.
- Whenever possible, liver samples of approximately 100 g should be cut off in such a way that they only present one cut surface.
- Perfuse with buffer A for 20 min at 37°C. Perfusion should be performed by several blunt-end cannulae inserted into vessels of the cut surface. The number of cannulae depends on the number of large vessels available on the cut surface. The flow should be adjusted to approximately 3 drops/s that leave the perfused tissue.
  Buffer A:
  - 498 mL washing buffer (8.3 g/L NaCl; 0.5 g/L KCl; 2.4 g/L HEPES; adjust to pH 7.4 with 4 *N* NaOH)
  - 2 mL of EGTA solution [ethylene glycol bis-(β-aminoethyl-ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid; Merck, Darmstadt, Germany)
- Perfuse with buffer B for 20 min at 37°C. Buffer B is identical to buffer A with the exception that no EGTA solution is added.
- Thereafter, perfuse with collagenase buffer for 30 min in a recirculating way at 37°C.
  Collagenase buffer:
  - Buffer C: 3.9 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, 0.7 g/L $CaCl_2 \cdot 2H_2O$; adjust to pH 7.2 by 4 *N* NaOH and prewarm to 37°C before use.
  - Dissolve 100 mg of collagenase (for instance: Sigma, C5138) in 200 mL of buffer C immediately before perfusion. Selection of an adequate batch of collagenase is the key critical step for successful isolation of human hepatocytes. Whereas the majority of all collagenases allows successful isolation of rat hepatocytes, selection of a good batch for human liver is more critical. Mostly, the collagenase concentration has to be optimized for an individual batch of collagenase.
- Transfer the tissue into a large Petri dish with suspension buffer (composition: see Table 8). Scrape liver cells gently out with a spatula.
- Filter the liver cell suspension through gauze.
- Centrifuge for 5 min at 50 × *g*.
- Wash twice with suspension buffer, centrifuge again, and resuspend in 30 mL suspension buffer.
- Determine Trypan Blue exclusion rate after 1:1 dilution of the hepatocyte suspension with a Trypan Blue solution (4 g/L Trypan Blue).

Note: The procedure described here is a modification of the technique described by Bader et al. [35].

TABLE 8

Standard Operation Procedure for Isolation of Rat Hepatocytes

The basic two-step collagenase perfusion technique has been described by Seglen [36]. Thus, the basic preparation techniques are not repeared here.

All buffers that come into contact with hepatocytes should be prewarmed to 37°C and should be carbogen-equilibrated before and during preparation.

- Anesthesize the rat (for instance, a male Sprague–Dawley rat, 180–280 g) with an i.p. injection of pentobarbital (60 mg/kg).
- Perfuse the liver *in situ* via the vena portae for 15 min with EGTA buffer at 37°C.
  EGTA buffer:
    - 124 mL glucose solution (9 g D-glucose/L)
    - 20 mL KH buffer (60 g NaCl/L, 1.75 g KCl/L, and 1.6 g KH_2PO_4/L; adjusted to pH 7.6 with NaOH)
    - 20 mL HEPES buffer (60 g HEPES/L; adjusted to pH 8.5 with NaOH)[a]
    - 30 mL amino acid solution (0.27 g/L L-alanine, 0.14 g/L L-aspartic acid, 0.4 g/L L-asparagine, 0.27 g/L L-citrulline, 0.14 g/L L-cysteine, 1.0 g/L L-histidine, 1.0 g/L L-glutamic acid, 1.0 g/L L-glycin, 0.40 g/L L-isoleucine, 0.8 g/L L-leucine, 1.30 g/L L-lysine, 0.55 g/L L-methionine, 0.65 g/L L-ornithine, 0.55 g/L L-phenylalanine, 0.55 g/L L-proline, 0.65 g/L L-serine, 1.35 g/L L-threonine, 0.65 g/L L-tryptophan, 0.55 g/L L-tyrosine, 0.80 g/L L-valine; dissolve amino acids that cannot be dissolved at neutral pH by the addition of 10 *N* NaOH and thereafter adjust to pH 7.6 by 37% HCl)
    - 2 mL glutamine solution (7 g L-glutamine/L; freshly prepared) 1.0 mL insulin solution (2 g insulin/L dissolved in 1 *N* NaOH, adjusted to pH 7.6 by 1 *N* HCl; freshly prepared)
    - 0.8 mL EGTA solution (47.5 g EGTA/L; dissolved by addition of NaOH, adjusted to pH 7.6 by HCl)
- Continue perfusion for 30 min with the collagenase buffer (37°C)
  Collagenase buffer:
    - 155 mL glucose solution
    - 25 mL KH buffer
    - 25 mL HEPES buffer
    - 38 mL amino acid solution
    - 1.2 mL insulin solution
    - 10 mL $CaCl_2$ solution (19 g $CaCl_2 \cdot 2H_2O$/L)
    - 2.5 mL glutamine solution
    - 125 mg collagenase (for instance, type CLSII; 255 U/mg; Biochrom (Berlin, Germany); dissolve collagenase in the prewarmed mixture of the above-mentioned solutions immediately before use

The flow for the EGTA and collagenase buffers should be ~10 mL/min and the pressure of perfusion should not exceed a 20-cm water column.

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TABLE 8 (Continued)

- After perfusion, remove the liver from the animal, remove the liver capsula, and dissociate carefully in suspension buffer
  Suspension buffer:
  - 620 mL glucose solution
  - 100 mL KH buffer
  - 100 mL HEPES buffer (adjusted to pH 7.6; wthout carbogen-equilibration)
  - 150 mL amino acid solution
  - 10 mL glutamine solution
  - 5 mL insulin solution
  - 8 mL $CaCl_2$ solution
  - 4 mL $MgSO_4$ solution (24.6 g/L $MgSO_4 \cdot 7H_2O$)
  - 2 g BSA/L; dissolve in the mixture of the above-mentioned solutions
- Filter the liver cell suspension through gauze (100 μm pore size).
- Centrifuge for 5 min at 50 × *g*.
- Wash twice with suspension buffer, centrifuge again, and resuspend in 30 mL suspension buffer.
- Determine Trypan Blue exclusion rate after 1:1 dilution of the hepatocyte suspension with a Trypan Blue solution (4 g/L Trypan Blue).

[a] When HEPES buffer is used carbogen-equilibrated at 37°C, it should be adjusted to pH 8.5 before use. When HEPES buffer is used without carbogen equilibration, it should be adjusted to pH 7.6 before use.

cells will attach and form a viable monolayer on Petri dishes. This seems unproblematic for rat hepatocytes, but it would render the experiments expensive if the technique is used with commercially available human hepatocytes.

Recently, two encouraging studies on enzyme induction in cultures with cryopreserved hepatocytes have been published [30,31]. Protein expression of CYP1A1/2, 2B1/2, and 3A1/2 determined by Western blot analysis was induced in monocultures with cryopreserved rat hepatocytes by phenobarbital and dexamethasone in a manner indistinguishable from that of fresh hepatocytes [31]. However, induction of CYP3A activity by dexamethasone measured as testosterone 6β-hydroxylase activity was reduced to ~68% in cryopreserved hepatocytes preincubated with carbogen for 30 min at 37°C and reduced to ~20% in hepatocytes without preincubation before cryopreservation [31]. Thus, preincubation improved enzyme induction in Silva's study [31], in contrast to our study, in which preincubation improved attachment efficiency but did not substantially alter induction of EROD, PROD, as well as regioselective and stereoselective hydroxylation of testosterone. This discrepancy might be caused by different cryopreservation protocols, because Silva et al. [31] applied a stepwise freezing protocol, placing cryovials at −20°C for 60 min followed by −70°C for another 60 min, whereas we used a computer-controlled freezing protocol, including a shock cooling step that avoids cell damage caused by the release of the latent heat of fusion during freezing [10]. Very encouraging results were also reported by Madan et al. [30]. Inducibility of CYP1A, 2B, 3A, and 4A was similar in monocultures prepared with cryopreserved and freshly isolated rat hepatocytes [30]. A computer-controlled freezing protocol was applied also in Madan's study, including a

TABLE 9

Standard Operation Procedure for Cryopreservation and Thawing of Hepatocytes

Cryopreservation

- Adjust hepatocytes to 3 Mio cells/mL in suspension buffer (composition: see Table 8) in an Erlenmeyer flask. Incubate for 30 min at 37°C during gentle shaking and carbogen equilibration.
- Determine the volume (original volume) and centrifuge the suspension for 5 min, 50 × *g*, 4°C. All further steps are performed at 4°C.
- Discard a volume equal to two-thirds of the original volume from the supernatant and resuspend the cell pellet in the remaining suspension buffer (one-third of the original volume) by shaking gently.
- Add ice-cold suspension buffer containing 12% (v/v) of DMSO to the cell suspension up to 50% of the original volume, resulting in a DMSO concentration of 4% and ~6 Mio/mL hepatocytes.
- After 5 min on ice, add suspension buffer containing 16% (v/v) of DMSO up to the original volume of the cell suspension, resulting in a DMSO concentration of 10% and 3 Mio hepatocytes/mL.
- After 5 min on ice, transfer the hepatocyte suspension to cold cryovials with 1.5 mL per vial.
- Start the freezing program within 5 min. The time period between the second addition of DMSO and initiation of cryopreservation should not exceed 10 min.
- The freezing procedure can be performed by any computer-controlled freezing machine (for instance, BV-8, Consarctic, Schöllgrippen, Germany). The freezing protocol should be performed as follows:
    - Cooling in 10 min down to 0°C
    - 8 min at 0°C
    - In 4 min down to −8°C
    - In 0.1 min down to −28°C
    - In 2 min down to −33°C
    - In 2 min up to −28°C
    - In 16 min down to −60°C
    - In 4 min down to −100°C

  The temperature in the chamber and in one cryovial should be monitored by a chart record to control whether crystallization heat was sufficiently compensated.
- Transfer the cryovials into liquid nitrogen immediately after the freezing program has been finished.

Thawing

- Thaw the frozen hepatocytes quickly by gentle shaking in a 37°C water bath. The hepatocytes should thaw, but not become warm.
- Transfer the hepatocyte suspension into ice-cold Erlenmeyer flasks immediately after thawing and dilute DMSO gradually by the addition of cold not carbogen-equilibrated suspension buffer, 0.5-, 1-, 2-, and 3-fold the volume of the thawed hepatocyte suspension. Suspension buffer should be added dropwise and hepatocytes should be 3 min on ice before the next dilution step takes place.
- After centrifugation (4°C, 50 × *g*, 5 min) and resuspension in 10 mL suspension buffer, the hepatocytes can be purified by Percoll centrifugation.

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TABLE 9 (Continued)

Percoll centrifugation

- Add the following into an ice-cold 50-mL Falcon tube:
  (a) 10 mL of hepatocytes in suspension buffer (containing maximally 20 Mio hepatocytes)
  (b) 22 mL of suspension buffer (without carbogen equilibration)
  (c) 18 mL of Percoll solution (Sigma)
- Mix gently and centrifuge at 250 × *g*, 4°C, for 20 min.
- After centrifugation, the cell pellet contains the intact hepatocytes. Resuspend the cell pellet in suspension buffer and wash twice in suspension buffer (centrifugation at 50 × *g*, 5 min, 4°C).

Incubation with test substances in suspension

- Transfer 1 mL of suspension buffer containing 1 Mio hepatocytes into glass vials. The diameter of the hepatocyte suspension in the glass vial should be at least twice its height.
- Transfer the glass vials into a shaking water bath or into an air-conditioned rotation incubator (36–37°C; ~40 rpm).
- Incubate up to 8 h. However, activities of xenobiotic metabolizing enzymes ≥60% of freshly isolated hepatocytes are guaranteed only up to 2 h.

shock cooling step to compensate for release of the latent heat of fusion and no preincubation with carbogen was included.

In conclusion, EROD, PROD, and testosterone hydroxylation activities could be induced in cultures with cryopreserved hepatocytes. Induction factors of all testosterone-hydroxylation products were similar in cultures with freshly isolated and cryopreserved hepatocytes. In addition, the induction pattern of the single regioselective and stereoselective testosterone hydroxylation products in cryopreserved cultures was similar to that of rats in vivo. However, in contrast to the induction factors, the absolute activities were lower in cryopreserved versus fresh cultures. Thus, before the presented culture system with cryopreserved hepatocytes can be applied to human hepatocytes and validated with further enzyme inducers, we plan to compare its performance with that of other systems using cryopreserved hepatocytes, such as hepatocytes immobilized in calcium alginate beads [32], cryopreserved complete monolayer cultures [33], and cryopreserved collagen sandwich cultures [34].

IX. CONCLUSIONS

Highly reproducible techniques have been established for the cryopreservation of hepatocytes from human, monkey, dog, rat, and mouse. Detailed descriptions of the hepatocyte isolation techniques are given for human (Table 7) and rat (Table 8). Techniques for monkey and dog are similar, although not identical, to the human isolation technique. The isolation procedure for rat hepatocytes is similar to that of the mouse. The same

protocol for cryopreservation and thawing (Table 9) can be applied for all species. Hepatocytes isolated and cryopreserved by our standard operation procedures (Tables 7–9) have a viability ≥80% and the metabolic capacity of phase I and phase II xenobiotic metabolizing enzymes is >60% of freshly isolated cells. Thus, experiments with hepatocytes can be performed without the need for fresh liver tissue. Cryopreserved hepatocytes in suspension can be recommended for short-term metabolism studies, for toxicity tests, or as metabolizing systems in mutagenicity tests. Incubation periods should not exceed 8 h. Studies on enzyme induction can also be performed with cryopreserved hepatocytes, but the technique requires further optimization.

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